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Short Communication

THE ROLE OF PLASMID CONSTRUCTS CONTAINING THE SV40 DNA NUCLEAR-TARGETING SEQUENCE IN CATIONIC LIPID-MEDIATED DNA DELIVERY

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Abstract: One of the steps that limit transfection efficiency in non-viral gene delivery is inefficient nuclear import of plasmid DNA, once it has been delivered into the cytoplasm. Recently, via microinjection into the cytoplasm and *in situ* hybridizations into a few cell types, it was shown that a region of Simian virus 40(SV40), specifically a c. 372-bp fragment of SV40 genomic DNA encompassing the SV40 promoter-enhancer-origin of replication (SV40 DTS), could enable the nuclear import of a plasmid carrying these sequences (Dean D.A. **Exp. Cell Res.** 230 (1997) 293). In this report, we address the issue of the suitability of the SV40 DTS for cationic lipid-mediated gene delivery, and its capacity to improve the efficiency of the transfection process. For this study, we used transient reporter gene expression assays on various cell types. The gene expression from the plasmid constructs carrying the SV40 DTS varied with cell type and plasmid construct used. Such cell-type and plasmid-construct dependency on gene expression from plasmids containing the SV40 DTS suggests that the gene expression from plasmids is not entirely dependent on its ability to enhance the nuclear import of said plasmids.

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Abbreviations used: β-gal - β-galactosidase; CLDC - cationic lipid DNA complexes; Chol - cholesterol; CMV - Cytomegalo virus; DHDEAB - *N*,*N*di-(*n*-hexadecyl)-*N*,*N*-di(hydroxyethyl)ammonium bromide; DTS - DNA nuclear targeting sequence; DMEM - Dulbecco's modified eagles medium; HEPES-HBSS - HEPES buffered Hank's balanced salt solution; EBNA1 - EBV nuclear antigen 1; EBV - Epstein Barr virus; luc or lux - Luciferase; NLS - nuclear localization sequence; NPC - nuclear pore complexes; PBS - phosphate buffered saline; SV40 DTS - SV40 promoter-enhancer-origin; SV40 - Simian virus 40; TNF-α – tumor necrosis factor-α; TPA - 12-O-tetradecanoyl-phorbol -13-acetate.

Key Words: Cationic Lipid, Gene Delivery, SV40 DTS, Nuclear Import, Gene Expression

INTRODUCTION

In non-viral gene delivery, in order to achieve efficient delivery of a gene, three major cell barriers have to be overcome [1]. First, the DNA should cross the plasma membrane, and then, it should escape from the endosomal membrane. Third, it should cross the nuclear membrane. The last step, referred to as nuclear import, is necessary for a gene to come into contact with the transcriptional machinery of a cell, which in turn is essential for that gene to be expressed. The nuclear import efficiency of a plasmid is considered to be an important kinetic block that influences transgenic expression in eukaryotic cells [2]. Virus-mediated transfections are considered to be more efficient due to the capabilities of viral proteins in crossing the nuclear membrane. However, in non-viral gene delivery, nuclear import is unaided and the level of transgenic expression obtained is relatively drastically reduced [3]. One attractive method to increase the nuclear import of plasmid DNA is to covalently attach a peptide sequence, known as NLS, to the DNA [4]. NLS, normally found with proteins that shuttle across the nuclear membrane through NPC are the subjects of extensive study [5]. Proteins containing NLS are transported into the nucleus through interaction with one or more of a broad family of NLS receptors, termed importins or karyophirins [6]. Recently, certain DNA sequences have been shown to help in the nuclear import of plasmid DNA, once it is in the cytosol. When the EBV oriP-containing plasmid was transfected into cells stably expressing EBNA1, an up to 100-fold increase in luciferase expression occurred [7]. This enhancement in gene expression was attributed to the increased efficiency of the nuclear import of DNA upon binding to NLS containing EBNA1. Chan and Jans [8] showed that delivering DNA with modified GAL4 protein, a transcription factor, can efficiently transport DNA to the nucleus from the cytoplasm. In this case, the NLS provided by the GAL4 helps in ferrying DNA across the nuclear pore. Incorporation of NFkB sequence was also shown to increase the nuclear uptake of the plasmid DNA upon induction with an inducer, TNF α or TPA [9].

Dean [10] reported that a region of the SV40 DNA has the unique ability to transport plasmids from the cytoplasm to the nucleus actively. This region is called the DTS and it is about 372 bp in length. This sequence was shown to help in the nuclear import of plasmids in various cell types when protein-free DNA was microinjected and detected via ***in situ*** hybridization. This SV40 region includes the SV40 early and late promoters along with two 72-bp repeat enhancers and the origin of replication. Although this full-length sequence was shown to be as efficient as the whole SV40 genomic DNA in transporting plasmid into the nucleus, a single 72-bp repeat was found to retain most of the nuclear import activity. The SV40 DTS was shown to exhibit an orientation- and position-independent nuclear import function [11], and was also shown to

increase the expression of the gene in muscle tissue [12] and a few other cell types [13]. With the objective of improving the level of gene expression/efficiency of nuclear import in non-viral DNA delivery, we tested the influence of the SV40 DTS in three different eukaryotic expression vectors in four different cell types by means of transient transfection experiments.

MATERIALS AND METHODS

pCMVβ-gal SPORT and Lipofectamine2000 were purchased from Invitrogen, USA. pGL3-control, pGL3 basic and pBlueScript were obtained from Stratagene. pECFP-C1 was purchased from Clontech. Plasmid pCH110 and T4 DNA ligase was obtained from Amersham Pharmacia. T4 DNA polymerase was obtained from New England Biolabs. α^{32}P-dATP was purchased from Bhaba Atomic Research Centre, Mumbai, India. Qiagen DNA purification columns were purchased from Qiagen. NBD-PE was purchased from Molecular Probes (USA). All the primers used in this study were synthesized in-house using the Applied Biosystems 394 DNA/RNA synthesizer. All the other chemicals were obtained from Sigma Co, USA.

Construction of plasmid vectors

pSV40enCMVβ-gal and pSV40DTSCMVβ-gal were constructed as follows: first, the SV40 enhancer and SV40 promoter-enhancer-origin (SV40 DTS) were respectively amplified from the pGL3 control and pCH110 using specific primers, and cloned in the Sma I site of pBlueScript to get pBSSK-SV40en and pBSSK-SV40DTS. The SV40 enhancer and SV40DTS fragments were then removed from pBlueScript by digestion with BamH I and Hind III, and then cloned in their respective sites in pCMVβ-gal-SPORT to get pSV40enCMVβ-gal and pSV40DTSCMVβ-gal, respectively. The sequences were confirmed by automated sequencing and restriction digestion. By replacing the Kpn I and Xba I fragments of pCMVβ-gal-SPORT and pSV40DTSCMVβ-gal, containing the β-galactosidase gene, with the Kpn I and Xba I luciferase gene fragment from pGL3 basic, we constructed pCMV luc and pSV40DTSCMV luc, respectively. pCMV luc DTS was constructed by replacing the Nhe I and BamH I ECFP gene fragments of pECFP-C1 with the Nhe I and BamH I luciferase gene fragments from pGL3 basic. pCMV luc ΔSV40 was derived from pCMV luc DTS by partial digestion with Stu I and Ssp I to remove a 300-bp SV40 sequence, followed by re-ligation. Plasmid constructs pCMV Lux ΔSV40 and pCMV Lux en were provided by David A Dean. All the plasmid constructs used in this study were prepared using Qiagen midi prep columns according to the manufacturer's protocol. They were quantitated by 260 nm absorbance, and the plasmid quality was checked on 1% agarose gel at a 260 nm/280 nm ratio. The description of all the plasmid DNA constructs used is shown in Tab. 1.

Cell culture and transient transfections

All the cell lines used were maintained in DMEM, containing 10% fetal calf serum and antibiotics (Penicillin, Streptomycin and Kanamycin) in an incubator at 37°C and 5% CO_2. Transient transfections were done as described before, using DHDEAB:Chol (1mM:1mM), whose synthesis, liposome preparation and characterization have been described [14], or Lipofectamine2000 according to the manufacturer's protocol. Transfections were done by complexing plasmid DNA and lipid at 1:1 lipid:DNA charge ratios. A β-galactosidase assay was done, as described before, after 24 h of transfection [14]. β-galactosidase activity was calculated from a standard graph constructed for a commercial β-galactosidase enzyme. β-galactosidase activity was normalized against a mlligram of cell protein. The transfection of aphidicolin-treated cells was done as described above, except that the cells were treated with a 5 μg/ml final concentration of aphidicolin in 10% serum-containing medium 24 h before transfection and 21 h after the complexes were removed from the cells. Each assay was performed in triplicate. The reported data is representative of three completely independent experiments.

Nick translation of plasmid DNA and the kinetics of cell association and plasmid uptake

pCMV β-gal, pSV40enCMV β-gal and pSV40DTSCMV β-gal were labeled with α-P^{32}-dATP by nick translation. 2.5 μg of each plasmid was used for nick translation. Nick translation was carried out according to the standard protocol [16]. For the plasmid uptake kinetics experiment, labeled plasmids were mixed with their respective cold plasmids such that each well of a 96-well plate should have about 10,000 counts and 0.3 μg of plasmid. Transient transfections were done as before. 1 h, 2 h, 3 h and 4 h after the transfection medium was removed, the cells were washed thrice with PBS and lysed in 50 μl of lysis buffer for 10 min. at room temperature. The percentage cell associated counts were then plotted as a function of time.

Quantitating internalized CLDC by Fluorescence Activated Cell Sorting (FACS)

To accurately quantitate the amount of plasmid DNA internalized by cells as CLDC, we prepared CLDC using DHDEAB:Chol (1:1 mol/mol) liposomes containing 5 mol% NBD-PE as a fluorescent marker. COS-1 cells were grown in 6-well plates to about 70%-80% confluency. Complexes were prepared with three different plasmids – pCMV β-gal, pSV40enCMV β-gal and pSV40DTS CMV β-gal at a 1:1 lipid:DNA charge ratio. Complexes containing about 1 μg of plasmid DNA were added to each well and incubated for about 3 h. After the incubation, cells were washed once with HEPES-HBSS. Cells were trypsinized and washed with HEPES-HBSS. The external fluorescence contributed by cell surface associated CLDC was quenched by adding 0.4% trypan blue containing HEPES-HBSS. The addition of trypan blue has been shown to efficiently quench

the external fluorescence of CLDC [17-19]. Cells were then washed twice with HEPES-HBSS without trypan blue, and finally resuspended in the same buffer. Samples were run on a FACS Calibur, BD Biosciences, and 10,000 events were collected per sample. Cells without the addition of CLDC were run as a control. The data is expressed as the percentage of cells that were positive for CLDC uptake.

FACS analysis of aphidicolin-treated cells

CHO cells were grown in 35 mm dishes till they reached a confluency of about 60%. The cells were then washed with PBS and 2 ml of DMEM containing 10% serum was added, which either contained aphidicolin (5 μg/ml) or contained only DMSO of an appropriate concentration (control). The cells were incubated for 24 h at 37°C and 5% CO_2. After 24 h, the medium was removed and the cells were washed twice with PBS. The cells were collected by trypsinization and washed with PBS to remove trypsin, and then were resuspended in cold 70% ethanol and stored at 4°C until they were processed for FACS analysis. The cells were washed twice with PBS and finally resuspended in about 0.8 ml of PBS. To this, about 0.1 ml of RNAse (1 mg/ml) and 0.1 ml of propidium iodide (400 μg/ml) were added, and tube was incubated at 37°C for 30 min. Samples were run on a FACS Calibur, BD Biosciences, and 10,000 events were collected per sample in a propidium iodide channel.

RESULTS AND DISCUSSION

For convenience, the details of all the plasmid DNA constructs used are shown in Tab. 1. Initially, we used three plasmid vectors – pCMV β-gal (pCMV β-gal-SPORT), pSV40enCMV β-gal and pSV40DTSCMV β-gal. pCMV β-gal is a control plasmid without SV40 DTS. pSV40enCMV β-gal includes only a SV40 72-bp repeat enhancer, hereafter referred to as partial SV40 DTS, and pSV40DTSCMV β-gal includes the SV40 promoter-enhancer-origin of replication, hereafter referred to as full-length SV40 DTS. We performed transient transfections in dividing COS-1, HeLa, CHO and NIH-3T3 cell types using a DHDEAB:Chol (1:1) cationic liposome formulation.

We observed a 2.5-fold increase in gene expression in the COS-1 cells given pSV40DTSCMV β-gal over its control plasmid, pCMVβ-gal (Fig. 1A). pSV40enCMV β-gal, which contains the 72-bp repeat enhancer sequence, did not give rise to any increase in expression compared to pCMV β-gal. There was no increase in gene expression with either pSV40enCMV β-gal or pSV40DTSCMV β-gal over the control plasmid pCMV β-gal, in CHO, HeLa and NIH-3T3 cells (Figs. 1B, C and D). In case of COS-1 and NIH-3T3 cells, pSV40enCMV showed less reporter gene expression compared to the control plasmid, pCMV β-gal. Transfections were repeated with Lipofectamine2000, a commercial transfecting agent, on COS-1 and NIH-3T3 cells with the three plasmids. There were no distinguishable differences in the data on transfection

efficiencies for the three plasmids between Lipofectamine2000 and DHDEAB:Chol, indicating that the behavior of the plasmid is not dependent on the transfecting lipid (data not shown).

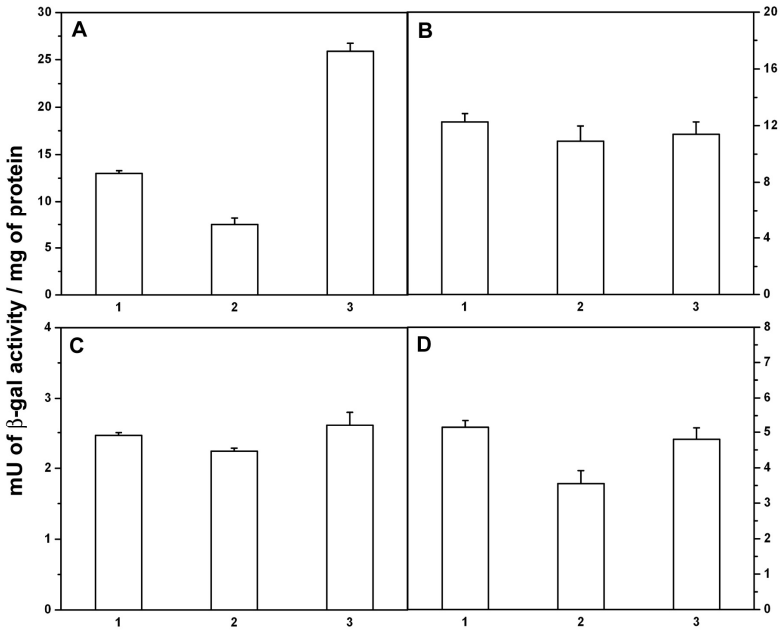

Fig. 1. Transient transfections with plasmid constructs. This panel shows plots of normalized milliUnits of β-galactosidase ativity from pCMV β-gal (1), pSV40enCMVβ-gal (2) and pSV40DTSCMVβ-gal (3) plasmid constructs in COS-1 (A), CHO (B), HeLa (C) and NIH-3T3 cells (D).

In order to see if the differential expression seen for pCMV β-gal, pSV40enCMV β-gal and pSV40DTSCMV β-gal in COS-1 cells is due to differential cell association and uptake of these plasmid constructs, we quantitated the kinetics of plasmid cell association and uptake as described above in the Materials and Methods section. Fig. 2A shows that all three plasmids were taken up to the same extent by COS-1 cells after transient transfection. We also carried out uptake experiments by quantitating only internalized complexes in COS-1 cells, as described in the Materials and Methods section. Fig. 2B shows that the uptake efficiency for the different plasmids is the same. We observed about 49.67%, 53.95% and 48.79% uptake efficiency for the pCMV β-gal, pSV40enCMV β-gal and pSV40DTS CMV β-gal plasmids, respectively. These experimental results indicate that the differences in the level of gene expression between these plasmid constructs is not due to differential internalization of these plasmids by the cells.

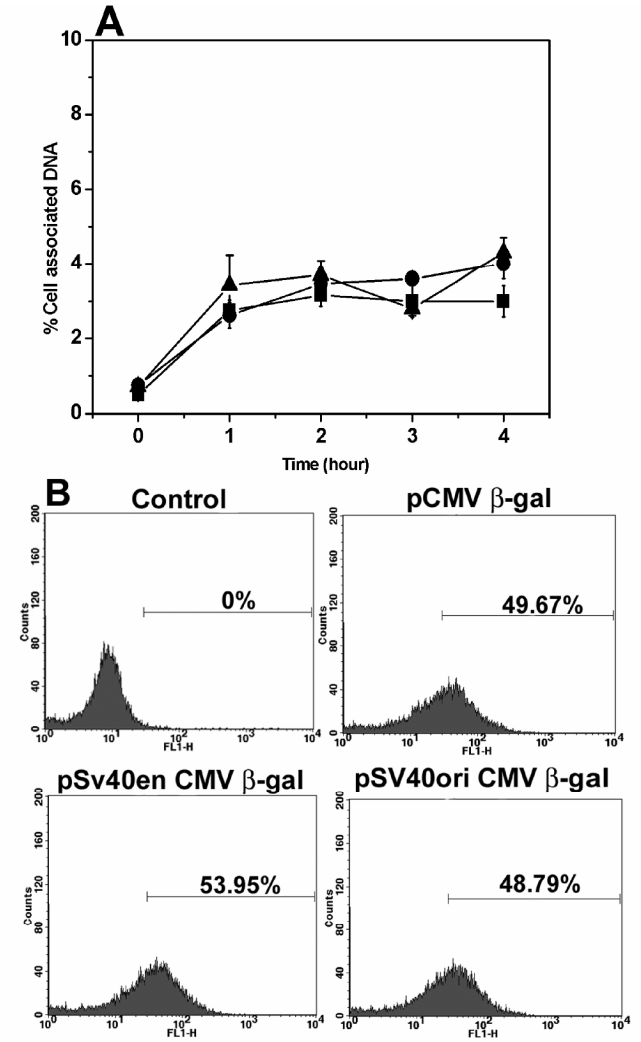

Fig. 2. DNA uptake studies in COS-1 cells. A - inetics of DNA cell association and uptake in COS-1 cells: the graph depicts the time course of cell association and the uptake of pCMV β-gal (¢), pSV40enCMVβ-gal (˜), and pSV40DTSCMVβ-gal (p) in COS-1 cells. B - Quantitation of internalized CLDC in COS-1 cells, assessed using FACS.

We also found that the transfection efficiency of these plasmid constructs was the same in COS-1 cells by co-transfecting the above constructs with a luciferase reporter gene plasmid (data not shown). Plasmid containing the SV40 origin of replication undergoes replication in COS-1 cells, which constitutively express the T-antigen [20]. Since pSV40DTSCMV β-gal contains the intact SV40 origin

of replication, the increase in gene expression seen only from pSV40DTSCMV β-gal and not from pSV40enCMV β-gal in COS-1 cells raised the possibility of plasmid replication playing a role in increasing the plasmid copy number, resulting in increased gene expression. To discriminate enhanced replication of plasmid from enhanced nuclear import of plasmid as the reason for increased reporter gene expression, we treated the cells with aphidicolin, a DNA polymerase α inhibitor [21]. We then performed transient transfections under this condition of replication blocking. β-galactosidase expression from pSV40DTSCMV β-gal was reduced seven-fold in the presence of aphidicolin, while the reporter gene expression from pCMV β-gal and pSV40enCMV β-gal was only reduced by 50% (Fig. 3). The significant decrease from pSV40DTSCMV β-gal suggests replication of the plasmid may be contributing to the higher reporter gene activity in COS-1 cells.

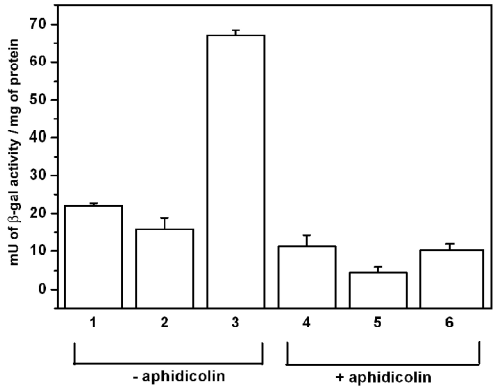

Fig. 3. Blocking of plasmid DNA replication. The figure shows the normalized milliUnits of β-galactosidase activity in COS-1 cells after transient transfections done with pCMV β-gal (1, 4), pSV40enCMVβ-gal (2, 5) and pSV40DTSCMVβ-gal (3, 6) either in the absence (1, 2 and 3) or in the presence (4, 5 and 6) of aphidicolin.

We tested the role of the plasmid backbone in the functioning of the SV40 DTS using three sets of plasmids. Each set of plasmids was derived from pECFP-C1 or pGL3 or pCMV-β-gal-SPORT. Each set consists of a plasmid backbone with or without SV40DTS. Luciferase was the reporter gene in all the plasmids. The pGL3-based vector contains a 72-bp enhancer of the DTS. The features of all the plasmid DNA constructs used in this study are shown in Tab. 1.

Transient transfections were again performed using these three sets of constructs with and without the SV40 DTS, in COS-1, CV-1, CHO and HeLa cells, treated with aphidicolin. Aphidicolin treatment arrests cells in S-phase [18], and the nuclear membrane is intact. We confirmed cell-cycle arrest in S-phase by aphidicolin treatment using fluorescence-activated cell sorter (FACS) (Fig. 4).

Tab. 1. Features of the plasmid constructs used in the study.

	Construct	Plasmid backbone	SV40 DTS	Reporter Gene
1	pCMV β-gal	pCMV-β galSPORT	Absent	β-galactosidase
2	pSV40en CMV β-gal	”	Partial	”
3	pSV40DTS CMV β-gal	”	Full	”
4	pCMV luc	”	Absent	Luciferase
5	pSV40DTS CMV luc	”	Full	”
6	pCMV luc ΔSV40	pECFP-C1	Absent	”
7	pCMV luc ΔSV40	”	Full	”
8	pCMV lux ΔSV40*	pGL3-enhancer	Absent	”
9	pCMV lux ΔSV40*	”	Partial	”

* Provided by David A Dean, Northwestern University, Chicago.

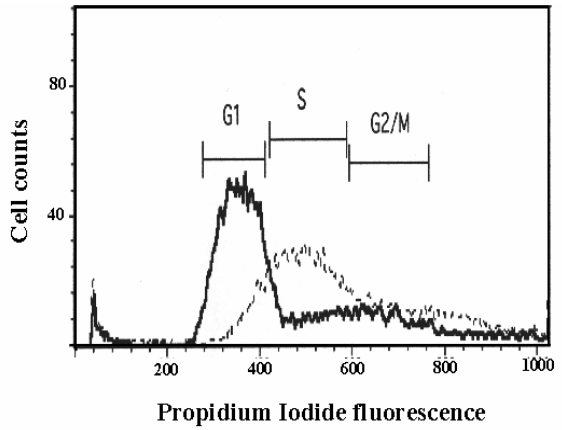

Fig. 4. FACS analysis of aphidicolin-treated CHO cells for S-phase cell-cycle arrest. The graph shows the FACS analysis of CHO cells, which were either untreated (——) or treated (– – –) with aphidicolin. 60% of the cells were found to have been arrested in S-phase after treatment, whereas in the control, only 18% of the cells were in S-phase.

Gene expression in S-phase arrested cells would primarily arise from plasmids that demonstrate the ability to enter into the nucleus. Expression from pCMV lux en was nearly 2-fold higher in COS-1 (Fig. 5A) and CHO cells (Fig. 5D) relative to its control plasmid (pCMV lux ΔSV40), while it did not show an increase in expression in HeLa cells (Fig. 5B). Since the variations in the percentage of internalized cells was less than 10%, the observed decreases in luciferase expression in HeLa cells with the plamids pCMV luc DTS and pCMV lux en are unclear and may be due to events subsequent to uptake, including interactions with cytoplamsic factors. Expression from pSV40DTSCMV luc showed a 2.5-fold increase relative to its control plasmid, pCMV luc, in CV-1 cells (Fig. 5c)

and has no effect in the other three cell types. Reporter gene expression from pCMV luc DTS was either decreased or marginally increased in all the cell types compared to its respective control plasmid, pCMV luc ΔSV40.

The presence of the SV40 DTS was shown to improve the efficiency of nuclear import pBR322, pUC19 and pGL3 basic plasmid, which do not otherwise have the ability to enter the nucleus [10]. The full-length SV40 DTS was shown to specifically enhance nuclear import by *in situ* hybridization of microinjected protein-free plasmid DNA [10]. However, similar SV40 DTS-mediated enhancement in gene expression in different cell types was not available. The gene expression results obtained in our study from different cell types blocked in S-phase and different plasmid constructs containing either full-length or partial SV40 DTS cannot simply be explained by the nuclear import property of SV40 DTS.

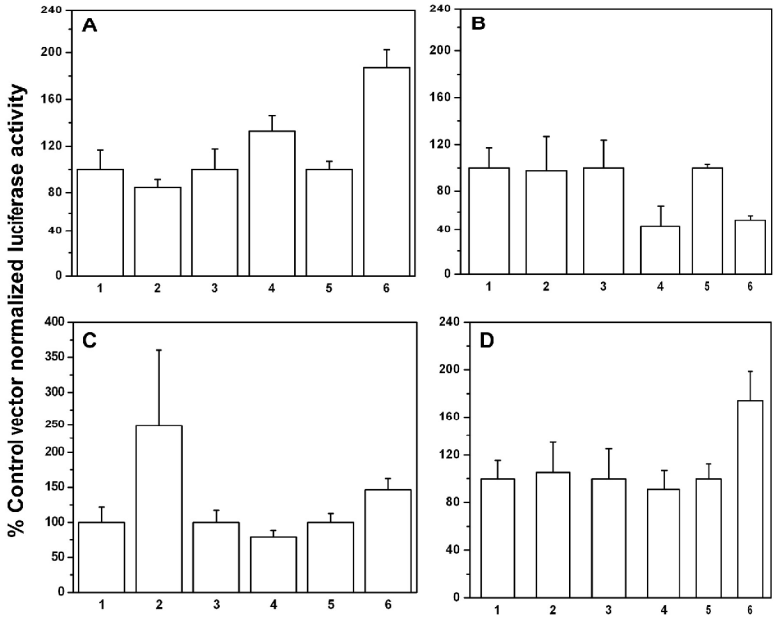

Fig. 5. Transient transfections with luciferase reporter plasmid constructs. This figure shows the normalized luciferase activity after transient transfection with pCMV luc (1), pSV40DTS CMV luc (2), pCMV luc ΔSV40 (3), pCMV luc DTS (4), pCMV lux ΔSV40 (5) and pCMV lux en (6) in COS-1 (A), HeLa (B), CV-1 (C) and CHO cells (D).

The underlying mechanism of SV40 DTS-mediated nuclear import was thought to be its ability to bind to a set of transcription factors which possess NLS. However, a comparison of other viral promoter and enhancer sequences revealed that simple binding of the transcription factor would not lead to nuclear import, but that the overall organization and structure of the transcription factor-DNA complex might be important (David A Dean's Northwestern University,

Chicago, personnel communication). This requirement would probably also influence the kinetics of the nuclear import of the SV40 DTS-containing plasmid, which lasts close to 8 h, whereas the SV40 virion takes only 60 min to reach the nuclear interior [22]. Studies carried out by other groups with the above SV40 sequence have not provided proof of a clear nuclear import role for this sequence. Earlier, it was shown that the deletion of certain enhancer motifs that are part of the 72-bp DTS did not reduce the level of nuclear localization of cytoplasmically injected plasmid DNA [23]. SV40 DNA was shown to require its capsid proteins for its efficient nuclear transport, whereas protein free SV40 DNA was shown to be inefficient in nuclear entry by microinjection experiments in TC7 cells [24]. In addition, the SV40 DTS can also act as a strong regulatory sequence of gene expression. The SV40 enhancer was shown to increase or decrease the level of gene expression from a gene either under its own promoter or under a heterologous promoter, such as mouse DNA polymerase β-gene promoter in a cell type-dependent fashion [25], or CMV promoter in COS-1 cells [26]. These findings and Dean's observations [10, 11] taken together suggest that the SV40 DTS might have multiple roles.

This study evidently demonstrates that incorporating the SV40 DTS into eukaryotic expression vectors containing a reporter gene under the CMV promoter-enhancer does not increase the level of gene expression in all the cell-types. Increase in gene expression from such plasmid constructs strongly seem to depend on the cell type and the plasmid construct used. This fact indicates that the gene expression, an end result of nuclear import, transcription and translation, is weakly dependent on the nuclear import property of the SV40 DTS alone. Our work shows that addition of the SV40 enhancer to the plasmids used for gene delivery is unlikely to be the solution that improves gene transfer efficiency. We conclude that the variable expression observed in different S-phase arrested cells could be due to the variable nuclear import activity of the SV40 DTS in a cell type- and plasmid construct-specific manner.

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